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Full Length Research Paper

Antioxidant activities and RP-HPLC identification of polyphenols in the acetone 80 extract of *Salvadora persica*

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***Salvadora persica* L. is one of the oldest known oral hygiene tools with high therapeutic effects on cariogenic bacteria and periodontal pathogens due to its well documented chemical constituents. In this paper, and for the first time, we report the use of RP-HPLC technique to identify the polyphenols in the acetone 80 extract obtained from stems of *S. persica*. The antioxidant properties (total antioxidant capacity, total phenolic and tannin content, DPPH, reducing power, and -carotene bleaching tests) of the extract were also investigated. Our results showed the presence of eleven compounds: caffeic acid (5.82%), rutin trihydrate (2%), trans-cinnamic acid (1.58%), gallic acid (1.53%), resorcinol (1.33%), chlorogenic acid (1.14%), quercetin dihydrate (0.67%), naphtho-resorcinol (0.48%), catechine hydrate (0.24%), *p*-coumaric acid (0.11%), and 3.4 dihydroxyphenylacetic acid (0.01%). The diluted acetone extract showed weak antioxidant activities comparatively to the BHT (DPPH IC_{50} 75 µg/ml; reducing power EC_{50}, 1940 $\mu g ml^{-1}$ and IC_{50}= 460 µg/ml for the -carotene bleaching test) . The total antioxidant activity was estimated at 0.528 mg GAE.g^{-1} DW, the total phenolic content was 0.443 mg GAE.g^{-1} DW and the total condensed tannin were 0.39 mg EC.g^{-1} DW. These results confirm the wide range of the biological activities of *S. persica* and reinforce the use of this plant of a natural source of antioxidant compounds with benefic properties for Human health.**

Key words: *Salvadora persica*, RP-HPLC, polyphenols, antioxidant activities.

INTRODUCTION

The species *Salvadora persica* L. belongs to the family Salvadoraceae (Andrews, 1956). This family also includes the species *Salvadora oleoides* that is used for chewing sticks in some areas in Africa and Western India. This tree is widely known as the "Arak" tree and is used by Arabs, Muslims and the North Africa natives and thus this chewing stick has more than one pronunciation. Such examples are "miswak" or "siwak». Arak has a wide geographical distribution over the Middle East and most of the African countries, example, Angola, Senegal, Ethiopia, Saudi Arabia, Sudan, Southern Egypt, Chad and Eastern parts of India (AbdElRahman et al., 2003; Ronse De Craene and Wanntorp, 2009).

The main use of *S. persica* is as a tool for teeth, tongue and gum cleaning and has also been used to treat toothache. This plant has large specter of biological activities including: antifungal, antibacterial, anti-inflammatory and hypoglycemic activities besides astringent and detergent effects. Regarding its different

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parts, *S. persica* has many uses in traditional medicine. The plant in various forms is applied to treat female sterility, gonorrhea, stomach-aches, spleen troubles, chest diseases, vesicle catarrah, rheumatism, liver diseases, ancylostomiasis, wounds, swollen joints, inflamed glands, cough, asthma and tremors, and as an ascarifuge for gastric troubles, and as vermifuge. These 'medicines' also have diuretic properties and influence lipid metabolism. They are even used as an antidote to all sorts of poison and have anti-scorbutic properties (AbdElRahman et al., 2003; Sofrata et al., 2008; Rajesh et al., 2009; Rajabalian et al., 2009; Al-Bayati et al., 2010; Noumi et al., 2010).

An indole alkaloid being an urea derivative, salvadoricine, from the leaves, salvadourea and glucotropaeolin, from the roots, 1-triacontanol and 1-octacosanol from the stems and trimethylamine from the rootbark have been isolated and characterized by chemical and spectral data. In these studies myristic, lauric and palmitic acids have been identified as the major components of the seeds. Trimethylamine was also isolated from the leaves and the bark and the presence of alkaloids has been reported. The leaves contain a flavonoid quercetin and many phenolic acids like vanillic, syringic, ferulic, p-hydroxybenzoic and salicylic acid. The presence of minerals, fluoride, silica, sulphur, sodium bicarbonate, chloride and calcium has been reported. Resins in small amount, tannins (tannic acid), alkaloids in large amounts, flavonoids, sterols, -sitosterol, m-anisic acid, salvadourea [1,3-Bis-(3-methyoxy- benzyl-) urea], essential volatile oils, example, mustard oil, vitamin C, and steam-distillable compound of 10% benzyl nitrate and 90% benzyl-isothiocyanate have all been reported from the roots. In addition to some of the above mentioned compounds, the root contains 27% ash, large amounts of chlorine, trimethylamine, but negligible amount of tannins and saponins. It is believed that some of these components are useful in tooth cleaning (Ahmed et al., 2008; AbdElRahman et al., 2002; El-Kholy et al., 2008; Rajesh et al., 2009; Sher et al., 2010; Manavalan et al., 2010).

The aim of the present work was to evaluate the antioxidant properties of acetone 80 extract of the stems of *S. persica* and to identify the polyphenols by using the RP-HPLC technique.

MATERIALS AND METHODS

Chemical and reagents

Folin- Ciocalteu reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid, sodium nitrite solution ($NaNO_2$), aluminum chloride hexahydrate solution ($AlCl_3$, $6H_2O$), vanillin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, iron(III)chloride anhydrous ($FeCl_3$), ascorbic acid and ß-carotene were purchased from Fluka (Buchs, Switzerland). Butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich (GmbH, Sternheim, Germany). Sulfuric acid (H_2SO_4) and Kalium-hexacyanoferrat (III); $K_3Fe(CN)_6$ were obtained from Merck (Darmstadt, Germany). All solvents used in this study were purchased from Sigma-Aldrich (GmbH, Sternheim, Germany).

Plant material extraction

Dry stems of *S. persica* imported from Morocco were purchased from a local market in the region of Sidi Bouzid (Tunisia). Extraction was made with a mixture of acetone: water (80:20; v/v). 250 g of dry stems powder were tested. The extract was prepared by adding 4 g of small particle fresh plant material powder with a commercially available food blender to 40 ml solvent and allowing the mixtures to stand overnight at room temperature, after which the supernatants were filtered and dried/evaporated under a controlled temperature (4°C). They were stored at 4°C until analysis (Trabelsi et al., 2010). To make 30 mg extract-impregnated disks, 1 ml of extract solution in diluted acetone was applied onto the sterile disks in 10 µl increments with sufficient time in between to allow drying.

Antioxidant properties

Phenolic compounds analysis

Evaluation of total antioxidant capacity: The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and sub-sequent formation of a green phosphate/Mo (V) complex at acid pH according to the protocol described by Prieto et al. (1999). An aliquot (0.1 ml) of plant extract was combined to 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95°C for 90 min. After, the mixture had cooled to room temperature; the absorbance of each solution was measured at 695 nm (Anthelie Advanced 2, SECOMAN) against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0 to 500 µg/ml. All samples were analyzed in triplicate.

Total phenolic content: Phenolic content was assayed using the Folin-Ciocalteu reagent, following Singleton's method slightly modified (Dewanto et al., 2002). An aliquot (0.125 ml) of appropriately diluted sample extract was added to 0.5 ml of distilled water and 0.125 ml of the Folin-Ciocalteu reagent. After 3 min, 1.25 ml of Na_2CO_3 solution (7 g/100 ml) were added and the final volume was made up to 3 ml with distilled water. The absorbance was measured at 760 nm, after incubation for 90 min at 23°C in dark. Total phenolic content of leaves was expressed as mg gallic acid equivalents per gram of dry weight (mg $GAE.g^{-1}$ DW) through the calibration curve with gallic acid. The calibration curve range was 0 to 400 $\mu g.ml^{-1}$. Triplicate measurements were taken for all samples.

Total condensed tannins: Proanthocyanidins were measured using the modified vanillin assay described by Sun et al. (1998).To 50 l of suitably diluted sample were added 3 ml of methanol vanillin solution and 1.5 ml H_2SO_4 respectively. The mixture was allowed to stand for 15 min at room temperature, and the absorption was measured at 500 nm against solvent as a blank. The amount of total condensed tannins is expressed as mg (+)-catechin g^{-1} DW.

Antioxidant activities

DPPH radical–scavenging activity

The antioxidant activity of different solvent extracts was measured in term of hydrogen donating or radical scavenging ability using the stable DPPH method according to the method described by Hanato

et al. (1988). The sample was diluted in pure solvent of extraction at different concentrations (10, 20, 100 and 200 µg.ml^{-1}), then 1 ml of each diluted plant extract was added to 0.25 ml of a 0.2 mmol/L DPPH methanolic solution. The mixture of different extract concentration and DPPH were placed in the dark at room temperature for 30 min. The absorbance of the resulting solution was then read at 517 nm. The antiradical activity was expressed as IC_{50} (µg.ml^{-1}). The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0-A_1)/A_0]*100$ (1)

where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

Determination of reducing power

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Oyaizu (1986). Briefly, 1 ml of *S. persica* extract was mixed with 2.5 ml of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1g/100 ml). After incubation at 50°C for 25 min, 2.5 ml of trichloroacetic acid (10 g/100 ml) was added and the mixture was centrifuged at 650 × g for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of aqueous $FeCl_3$ (0.1g/100 ml). The absorbance was measured at 700 nm. The mean of absorbance values were plotted against concentration and a linear regression analysis was carried out. Increased absorbance of the reaction mixture indicated increased reducing power. EC_{50} value (mg.ml^{-1}) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as positive control.

-carotene-linoleic acid model system (B-CLAMS)

The B-CLAMS method by the peroxides generated during the oxidation of linoleic acid at elevated temperature (Koleva et al., 2002). In this study the B-CLAMS was modified for the 96-well micro-plate reader. In brief, the B-carotene was dissolved in 2 ml of $CHCl_3$, to which 20 mg of linoleic acid and 200 mg of Tween 40 were added. $CHCl_3$ was removed using rotary evaporator. Oxygenated water (100 ml) was added, and the flask was shaken vigorously until all material dissolved. This test mixture was prepared fresh and using immediately. To each well, 250 µl of the reagent mixture and 35 µl sample or standard solution were added. The plate was incubated at 45°C. Readings were taken at 490 nm using visible/UV microplate kinetics reader (EL x 808, Bio-Tek instruments) All samples were prepared and analyzed in triplicate.

Identification of phenolic compounds using RP-HPLC

Phenolic compounds analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC) coupled with an UV-Vis multiwavelength detector using the same protocol previously described by Trabelsi et al. (2010). Five microgrammes of the stem extract was diluted in 1 ml of methanol (HPLC grade). The separation was carried out on 250 × 4.6 mm, 4 m Hypersil ODS C18 reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (Solvent A) and water with 0.2% sulphuric acid (Solvent B).The flow rate was kept at 0.5 ml/min. The gradient program was as follows: 15% A/85% B 0 to 12 min, 40% A/60% B 12 to 14 min, 60% A/40% B 14 to 18 min, 80% A/20% B 18 to 20 min, 90% A/10% B 20-24 min, 100% A 24 to 28 min. The injection volume was 20 l and peaks were monitored at 280 nm. Samples were filtered through a 0.45 m membrane filter before injection. Each experiment was repeated at least two times. Peaks were identified by congruent retention times compared with standards (Table 1).

RESULTS AND DISCUSSION

The results of the antioxidant properties of Acetone 80 *S. persica* extract are summarized in Table 2. The study reveals that the antioxidant activity of Arak extract was about 0.528 mg GAE.g^{-1} DW and the total phenolic content was 0.443 mg GAE.g^{-1} DW. Similarly, total condensed tannin content of diluted acetone extract was estimated at 0.39 mg EC.g^{-1} DW. The determination of the concentration corresponding to 50% of inhibition of radical DPPH showed that the IC_{50} of diluted acetone extract of *S. persica* was 75 µg/ml. In the reducing power assay, our results showed that the EC_{50} of *S. persica* diluted acetone extract (EC_{50}, 1940 µg.ml^{-1}) and largely different to the reducing power of the standard BHT (EC_{50}, 75 µg.ml^{-1}) . The antioxidant activity of diluted acetone *S. persica* extract measured by the bleaching of -carotene was estimated at IC_{50}= 460 µg.ml^{-1}.

Polyphenols are among the secondary metabolites that have significant antioxidant capacities added to the anti-allergenic, anti-inflammatory, anti-thrombotic, anti-mutagenic, and anticancer properties and cardioprotectrive and vasodilatory effects (Trabelsi et al., 2010). Phenolic compounds possess also a considerable antimicrobial capacity against a very large number of human pathogens.

The results of the identification of polyphenols by RP-HPLC (Figure 1) showed that the extract of *S. persica* (Acetone 80) is composed of 87 molecules. Indeed, the extract of Arak contains 28 compounds with a percentage varying from 1 to 22.3% among them there are: caffeic acid (5.82%), rutin trihydrate (2%), trans-cinnamic acid (1.58%), gallic acid (1.53%), resorcinol (1.33%), chlorogenic acid (1.14%), and 59 compounds with a percentage range of 0.039 to 0.94% among them there are: quercetin dihydrate (0.67%), naphtho-resorcinol (0.48%), catechine hydrate (0.24%), p-coumaric acid (0.11%), and 3.4 dihydroxyphenylacetic acid (0.01%).

In this paper, we report for the first time, the identification of polyphenols in the stems of *S. persica* identified using the RP-HPLC technique. Our results showed the presence of several compounds with known antioxidant properties and we noted that caffeic acid, rutin trihydrate, trans-cinnamic and gallic acids were the predominant compounds. In fact, all the identified polyphenols and flavonoids possess anti-inflammatory, anti-mutagenic, anticancer, antioxidant, anti-bacterial, anti-viral (an important anti-HIV capacity), anti-allergic, antihypertensive, anti-arthritic and many other biological activities (Trabelsi et al., 2010). Also, the flavonoids are potentially useful in the prevention of arteriosclerosis, cancer, diabetes, neurodegenerative diseases, arthritis and proanthocyanidins, which are a third important group

Table 1. Retention times of phenolic acid and flavonoid standards analysed by RP-HPLC and monitored at 280 nm.

S/No.	Standards	Retention time (min)
1	Gallic acid	5.68
2	3.4-Dihydroxybenzoic acid	8.1
3	3.4-Dihydroxyphenylacetic acid	8.16
4	Chlorogenic acid	8.58
5	Catechine hydrate	8.98
6	Cafeic acid	10.63
7	4-Hydroxybenzoic acid	10.65
8	3.5-Dimethoxy-4-hydroxybenzoic acid	10.89
9	Syringic acid	10.95
10	Rutine trihydrate	11.11
11	Vanillic acid	11.28
12	2.5-Dihydroxybenzoic acid	11.9
13	Naringin	12.89
14	Quercetine-3-rhamnoside	13.46
15	*p*-Coumaric acid	13.5
16	Trans-4-hydroxy-3-methoxycinnamic acid	14.09
17	Ferulic acid	14.19
18	Rosmarinic acid	15.25
19	Trans hydroxycinnamic acid	16.28
20	*o*-Coumaric acid	16.24
21	Luteolin	18.16
22	Quercetine dihydrate	18.28
23	Kaempferol	18.32
24	Naphtoresorcinol	18.44
25	Salycilic acid	18.68
26	trans cinnamic acid	19.2
27	Apigenin	19.27
28	Flavone	24.55
29	Coumarine	25.56
30	Carnosic acid	26.09
31	BHT	28.9

Table 2. Antioxidant activities of diluted acetone extract of *S. persica*.

Tests	***S. persica***	**BHT**
TAA: (mg GAE.g^{-1} DW)	0.528	-
Total Polyphenols: (mg GAE.g^{-1} DW)	0.443	-
Tannins : (mg EC.g^{-1} DW)	0.39	-
DPPH: IC_{50} ($\mu g.ml^{-1}$)	75	11.5
β- carotenes: IC_{50} ($\mu g.ml^{-1}$)	460	75
RP: EC_{50} ($\mu g.ml^{-1}$)	1940	75

TAA: Total antioxidant activity is expressed as mg gallic acid equivalents per gram of dry weight; total polyphenols is expressed as mg gallic acid equivalents per gram of dry weight; tannins is expressed as mg (+)-catechin/g DW; DPPH radical–scavenging activity is expressed as IC_{50} values (µg/ml); β-carotenes bleaching test is expressed as IC_{50} values ($\mu g.ml^{-1}$); RP: reducing power was expressed as EC_{50} values (µg/ml).

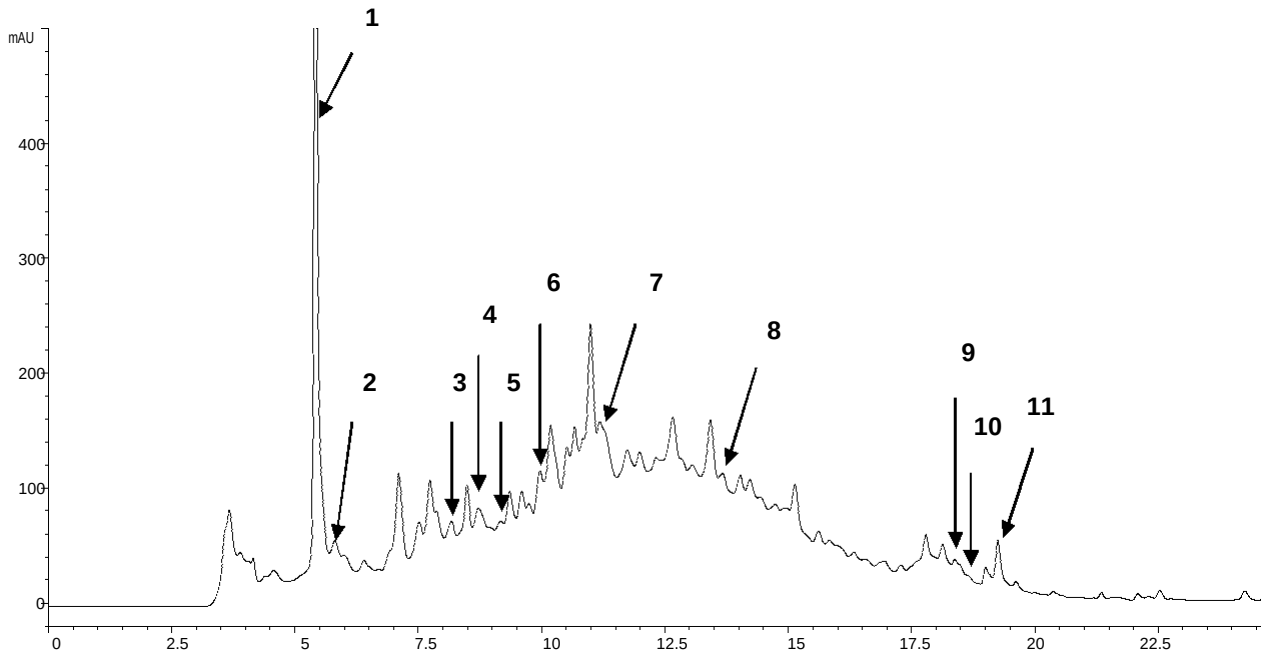

Figure 1. RP-HPLC Chromatographic profiles of phenolic acids and flavonoids in diluted acetone extract of *S. persica* stems monitored at 280 nm. The peak numbers correspond to:1, cafeic acid; 2, gallic acid; 3, 3.4 dihydroxyphenylacetic acid; 4, chlorogenic acid; 5, catechine hydrate; 6, resorcinol; 7, rutine trihydrate; 8, *p*-coumaric acid; 9, quercitine dehydrate; 10, naphto-resorcinol; 11, trans-cinnamic acid.

of phenolic compounds, are known to act against caries, reduce blood pressure and inhibit some physiological and receptor. The tannins (such as condensed tannins) have various therapeutic effects through their antibacterial, antiviral, anti-carcinogenic, anti-inflammatory and anti-allergic (Trabelsi et al., 2010).

CONCLUSION

In this paper, we report the identification of several polyphenols in the Acetone 80 extract of *S. persica* dry stems. Caffeic acid and rutine tryhydrate were the main components identified. Additionally to the known molecules with benefic interest for Human health described in *S. persica* (benzyl isothiocyanate, limonene, -pinene, Oleic and Iinoleic acids, Vitamin C, calcium, NaCl, KCl, trimethylamine, salvadourea and salvadorine…etc), the identified polyphenols in the present work contribute to highlight and reinforce the large scale of biological properties of this plant.

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